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(54) Title: CLONING OF ENTEROKINASE AND METHOD OF USE (57) Abstract Provided are nucleic acid sequences encoding enterokinase activity, the expression products thereof, and method for using same.		

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CLONING OF ENTEROKINASE AND METHOD OF USE

FIELD OF THE INVENTION

The present invention relates generally to the cloning and expression of enterokinase activity and to methods of its making and use.

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BACKGROUND OF THE INVENTION

The use of fusion proteins as a tool for recombinant protein production is well known in the biopharmaceutical industry. Fusing the coding sequence for a desired recombinant protein to that of a well-expressed gene has several advantages. Most fusion protein strategies position the protein of interest at the C-terminal end of the highly expressed fusion partner which allows translation initiation to occur on a "proven" gene sequence that is known to be well translated and can help ensure high expression levels. Some fusion partners can confer many advantageous attributes to the fusion protein, such as specific cellular localization, binding to affinity ligands to aid in purification and detection, and even proteolytic and conformational stability.

While fusion proteins offer numerous advantages, this beneficial physical association of the protein domains can also be problematic when it becomes necessary to separate the two (or more) components from their covalent tethering. The method of protein cleavage must be both specific and efficient and must not yield unwanted side products. This is particularly so when utilizing a fusion protein approach for the production of biopharmaceuticals destined for human use. Ideally, the most useful method allows for cleavage at a specific target sequence without regard for the internal protein sequence and/or without regard for the composition of the fusion partners. The method should produce cleaved product with authentic N- and C-termini, should not modify or otherwise adulterate the desired protein product, and should be tolerant to a wide range of conditions so that reaction components can be tailored to the physical characteristics of the fusion protein without seriously affecting the efficiency of the cleavage reaction. In addition, for biopharmaceutical production and applications, the cleaving reagent should not be from an animal source due to concerns about contamination by infectious agents.

An ideal choice for such a "universal" fusion protein cleaving method is use of the mammalian enzyme enterokinase (enteropeptidase). Enterokinase is the physiological activator of trypsinogen and cleaves with high specificity after the sequence (Asp₄)-Lys. Light *et al.*, J. Protein Chem. 10:475-480(1991). It is possible to engineer the fusion protein to include a linker DNA sequence encoding the amino acid sequence recognized by enterokinase. See for example, Bollen *et al.*, USPN 4,828,988 (May 9, 1988); Rutter, USPN 4,769,326 (September 6, 1988); and Mayne *et al.*, USPN 4,745,069 (May 17, 1988). However, although extensive research efforts have been mounted by several different research groups since the first partial purification of bovine enterokinase more than 15 years ago, no one has yet

been successful in cloning enterokinase. Porcine enterokinase was first isolated in the early 1970s (Maroux *et al.*, J. Biol. Chem. 246:5031(1971)) and bovine (Anderson *et al.*, Biochemistry 16:3354(1977)) and human (Grant *et al.*, Biochem. J. 155:243(1976)) enterokinases were isolated in the late 1970s. Liepnieks *et al.*, J. Biol. Chem. 254:1677(1979) described an enterokinase having 35% carbohydrate, a molecular weight of 150,000, with a heavy (115,000) and light (35,000) chain connected by one or more disulfide bonds. Subsequent studies of the light chain, *i.e.*, the catalytic subunit, were reported in Light *et al.*, J. Biol. Chem. 259:13195(1984). Most recently, Light *et al.*, J. Protein Chem. 10:475(1991), disclosed what was later proven to be an incorrect partial amino-terminal sequence for the catalytic subunit of bovine enterokinase. To date, it has been impossible to obtain recombinantly produced enterokinase activity and there continues to exist a need for such a product.

BRIEF SUMMARY

The present invention provides novel purified nucleic acid sequences encoding enterokinase activity. Specifically provided is mammalian enterokinase activity, including human and bovine enterokinase and comprising the nucleic acid sequence as set forth in SEQ ID NO:1, encoding the catalytic light chain, as well as portions of the heavy chain. The sequence comprises 2581 nucleotides and includes the catalytic domain, *i.e.*, nucleotides 1691 to 2398. A nucleotide sequence encoding this enterokinase activity and contained in the plasmid designated pEK-2/GI734 was deposited with the American Type Culture Collection (ATCC) on February 2, 1993 and accorded the accession number 69232. In a further embodiment, the invention comprises the expression products of the novel sequences having enterokinase activity.

Nucleic acid forms such as genomic DNA (gDNA), complementary DNA (cDNA), and DNA prepared by *de novo* chemical synthesis from nucleotides, as well as DNA with deletions or mutations, allelic variants and sequences that hybridize thereto under stringent conditions (or which would hybridize but for the redundancy of the genetic code) are also within the contemplation of the invention so long as they encode polypeptides having enterokinase activity as defined below. Also, forms which contain modifications of the catalytic site of enterokinase which may allow for alteration of the specific cleavage site recognized by the enzyme are included. Further provided are novel messenger RNA (mRNA) sequences corresponding to these DNA sequences.

Association of nucleic acid sequences provided by the invention with homologous or heterologous species expression control sequences, such as promoters, operators, regulators, and the like, allows for *in vivo* and *in vitro* transcription to the corresponding mRNA which, in turn, allows translation of proteins and related poly- and oligo- peptides, in large quantities, having enterokinase activity. In a presently preferred expression system of the invention, enterokinase encoding sequences are operatively associated with a regulatory promoter sequence allowing for transcription and translation

in a eukaryotic cell system to provide *e.g.*, enterokinase polypeptides having protease activity. The novel nucleic acid sequences may optionally encode both the heavy chain and the light chain of enterokinase, or the light chain alone which surprisingly still provides enterokinase activity. The enterokinase activity of the invention may be generated from one or more expression vector(s) each comprising one or more portions of the enterokinase activity, or, alternatively, the enterokinase activity can be generated from one or more expression vector(s) contained in one or more cell lines, each of which express all or a portion of the enterokinase activity. Thus, the heavy and light chains may be separately expressed in separate cell lines if desired. In addition, the enterokinase activity can be produced as a fusion protein, *e.g.*, using thioredoxin as the fusion partner. Optionally, the fusion partner can be all or part of yet another proteolytic enzyme, such as PACE, trypsinogen, and the like. Indeed, such an enterokinase fusion protein can contain an enterokinase cleavage site between the component protein domains, thereby allowing autocatalytic processing to separate the two domains and to yield mature, active enterokinase.

Incorporation of these sequences into prokaryotic and eukaryotic host cells by standard transformation and transfection processes, is also within the contemplation of the invention and is expected to provide useful enterokinase in quantities greatly in excess of those obtainable from tissue sources. The use of appropriate host cells provides for such post-translational modifications, *e.g.*, truncation, glycosylation, *etc.*, when needed to confer optimal biological activity on the expression products of the invention. Such appropriate host cells can include for example *E. coli*, CHO, yeast, and lepidoptera cells.

Novel protein products of the invention include those having the primary structural conformation (*i.e.*, amino acid sequence) of enterokinase comprising the sequence substantially as set forth in SEQ ID NO:2 and having enterokinase protease activity. A presently preferred embodiment comprises the amino acid sequence substantially as set forth in SEQ ID NO:2 and specifically comprising amino acids 564 to 798. Antibodies to such products are also provided.

Also provided by the invention are methods for cleaving fusion proteins utilizing the novel protein products of the invention. These protein products can include both heavy and light chains or can be solely light chain enterokinase activity. Light chain alone is a "soluble" form of the enterokinase activity and is devoid of the non-enzymatic heavy chain which is believed to act as a membrane anchor *in vivo*. Surprisingly, while this form (light chain alone) of enterokinase is a poorer enzyme on trypsinogen, it is much more effective on fusion proteins. Provided also is a production method wherein one of the fusion protein members is itself enterokinase activity, which, upon cleavage of the fusion protein domains at a strategically located enterokinase recognition site, yields additional enterokinase activity at each round of cleavage to cleave more fusion protein.

Methods and pharmaceutical compositions are also provided for treating digestive disorders associated with low levels of enterokinase activity by administering the novel protein products of the invention.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description thereof which includes numerous illustrative examples of the practice of the invention reference being made to the Sequence Listing wherein:

SEQ ID NO:1 provides the 2581 nucleic acid sequence and SEQ ID NO:2 provides the predicted amino acid sequence of a portion of the non-catalytic domain (heavy chain) and the entire catalytic domain (light chain) of bovine enterokinase. Light chain is encoded by nucleotides 1691 to 2398 (amino acids 564 to 798) and the C-terminal portion of enterokinase heavy chain extends from nucleotides 1 to 1690.

DETAILED DESCRIPTION

The present invention provides recombinantly produced enterokinase activity, as well as methods of making and methods of using enterokinase activity. As used herein, enterokinase activity means the capability of cleaving peptide or protein substrates at a specific site; for protein substrates, this is generally following the sequence (Asp)₄-Lys, or a similar sequence such as those described in Light *et al.*, Anal. Biochem. 106:199(1980); (a cluster of negatively charged amino acids followed by a positively charged amino acid). Typically, such activity is measured by activation of trypsinogen by cleaving the N-terminal propeptide (containing (Asp)₄-Lys) with enterokinase and subsequently assaying the amount of active trypsin generated using tosyl-arginine-methylester (TAME). See, *e.g.*, Maroux *et al.*, *supra*. Alternatively, enterokinase activity can be measured directly by incubating the enzyme with the peptide substrate Gly-(Asp)₄-Lys- β -naphthylamide and measuring the increase in fluorescence (excitation at 337 nm, emission at 420 nm) generated by cleavage and release of the β -NA (β -naphthylamide) moiety. See, *e.g.*, Grant *et al.*, Biochem. Biophys. Acta. 567:207(1979). Bovine enterokinase is also active on some trypsin substrates like TAME and BAEE (benzyl-arginine-ethyl-ester).

While it is generally assumed that optimal activity derives from holoenzyme, *i.e.*, the two-chain, heavy and light form of the enzyme, Applicants' invention also provides a proteolytic activity that derives solely from the light chain. Thus, as used herein, the term enterokinase activity does not require the presence of both heavy and light chains and can be derived solely from light chain.

Moreover, the chains or regions of the chains need not be the expression product of one vector, rather they can be separately and individually expressed. As used herein the terms co-transfection or co-expression are meant to include processes where the relevant nucleic acid sequences encoding the heavy and/or light chain(s) may be on a single or on one or more separate transfection or expression vector(s). Co-transfection and co-expression may employ one or more heavy and/or light chain

sequences or may employ sequences having deletions from and/or mutations in the sequences but which still encode an enterokinase activity as described above.

In one embodiment of the invention, the enterokinase activity is the protein encoded by the nucleotide sequence set forth in SEQ ID NO:1 and includes the mature catalytic domain, *i.e.*, nucleotides 1691 to 2398. As used herein, the term "a sequence substantially as" set forth in a SEQ ID NO is meant to encompass those sequences which hybridize to the sequence under stringent conditions as well as those which would hybridize but for the redundancy of the genetic code. Stringent conditions are generally 0.2 X SSC plus 0.1% SDS at 65°C. The terms "substantially duplicative" and "substantially corresponding" are meant to include those sequences which, though they may not be identical to those set forth in a SEQ ID NO, still result in expression product, proteins, and/or synthetic polypeptides that have enterokinase activity. Thus, using the nucleotide sequence as set forth in SEQ ID NO:1, DNA encoding enterokinase activity can be isolated and cloned from other sources as well using appropriate vectors, selectable markers and recombinant DNA techniques. The corresponding cDNA can be prepared from appropriate mRNA sources. Genomic DNA encoding enterokinase activity may also be obtained from a genomic library using a cDNA probe or oligonucleotide probes. Alternatively, an enterokinase activity-encoding DNA sequence may be prepared synthetically. The use of intron-less, *e.g.*, cDNA sequences, are preferred, as bacterial expression requires intron-less sequences. The sequence may also be modified appropriately for expression in bacteria as described, *supra*.

The present invention also provides a method for producing enterokinase activity preferably in non-glycosylated form. The method involves culturing a host cell, preferably bacterial, transformed with (*i.e.*, containing and capable of expressing) a DNA sequence encoding the enterokinase activity which is under the expression control of suitable transcriptional control sequences. The DNA sequence may encode both the heavy and light chains, or only light chain, or only as much as is required to result in the expression of enterokinase activity and may be deliberately designed to include preferred codons for expression in bacterial cells as is well known in the art. In the latter case, the resulting expression product of such deliberately designed DNA sequences may contain full length and may also contain a truncated, biologically active, mature peptide sequence encoding enterokinase activity, *e.g.*, light chain alone.

In another preferred method for expression of enterokinase activity, the DNA sequence encoding the catalytic domain of enterokinase is fused to a signal peptide (pre-region) and pro-region of a gene, such as the human PACE gene. PACE is a serine protease which cleaves after dibasic residues and is responsible for propeptide processing of a number of secreted proteins. When the PACE signal peptide (pre-region) and pro-region coding sequence is fused in-frame to the mature enterokinase light chain coding sequence and expressed in mammalian cells, *e.g.*, CHO cells, COS cells, BHK cells, and the like, the sequence is translated to produce a chimeric protein which is secreted and which is then

processed to remove the signal peptide thereby yielding pro-enterokinase; subsequent cleavage, by either endogenous or exogenous PACE, removes the pro-peptide from the N-terminus of the enterokinase and mature enterokinase activity is secreted into the conditioned medium. Optionally, as a source of PACE, this method may employ co-expression of a modified, soluble form of the PACE gene having the transmembrane domain of PACE deleted. See, for example, Hatsuzawa *et al.*, *J. Biol. Chem.* 267:16094(1992) for a description of soluble PACE and delineation of the pro-peptide portion of the protein. Other pre/pro regions can also be used to similar advantage in expressing enterokinase activity, for example, the pre/pro region of yeast Kex2 as described in Brenner *et. al.*, *Proc. Natl. Acad. Sci. U.S.A.* 89:922(1992), or the pre/pro region of trypsinogen as described in LeHeuron *et al.*, *Eur. J. Biochem.* 193:767(1990).

As used herein, the term "pro-protein" means a protein having attached to it a "pro" region; a "pre-pro-protein" has a "pre-pro" region attached to it. The "pre" region, or signal peptide, refers to the most N-terminal stretch of amino acids which target the remaining portion of the polypeptide to be translocated across a membrane, *e.g.*, the endoplasmic reticular membrane, and is usually subsequently cleaved by an endogenous signal peptidase.

The "pro" region is an intervening region between the signal peptide (pre-region) and the mature protein. This sequence may be responsible for enhancing some post-translational modifications; it may be necessary for proper folding, or it may act to inhibit the activity of the mature protein until it is removed post-translationally. The pro region is usually removed after signal peptide cleavage by an endoprotease. A "pre/pro" region is a combination of the "pre" region and the "pro" region as described above. More specifically, useful DNA constructs include fusions of DNA encoding enterokinase activity with the pre/pro region of trypsinogen. The signal peptide and the entire 8 amino acid pro region of bovine anionic trypsinogen (which includes an enterokinase recognition site) is fused to the amino terminus of the mature enterokinase catalytic domain. Yet another DNA construct involves fusion of the mature enterokinase catalytic domain to the C-terminus of *E. coli* thioredoxin, having an intervening spacer sequence encoding a known cleavage site such as an enterokinase cleavage site.

The DNA sequence encoding enterokinase activity may be inserted by conventional methods into an expression vector suitable for the desired host cell as is well known in the art. For bacterial or yeast production, the DNA sequence should not contain introns. For higher eukaryotic expression, it is not necessary to avoid introns, but cDNA sequences are preferred. Preferably for eukaryotic expression, the DNA sequence should contain a secretory leader sequence. The vectors should contain typical vector elements well known in the art including replication sites, selectable markers and transcriptional control sequences compatible with the chosen host.

Various strains of *E. coli* useful as host cells for the production of non-glycosylated, homogeneous enterokinase activity are also well-known in the art. A non-exclusive list of such strains

includes MC1061, DH1, RR1, C600hfl, K803, JA221, HB101, JM101 and various K12 strains, including the strain used in the Examples. Alternatively, other bacterial species may be used, including *B. subtilis*, various strains of *Pseudomonas*, other bacilli and the like.

Enterokinase activity may also be produced by heterologous expression of an enterokinase activity encoding sequence in mammalian cells. Enterokinase activity is thus obtainable in glycosylated form, that is, unless glycosylation is prevented. Where desired, glycosylation can be inhibited by tunicamycin or by site-directed mutagenesis of glycosylation sites, as is well known in the art. Suitable mammalian expression vectors and host cells for production of enterokinase activity are also well known in the art and include, without limitation, the vectors pXM and pMT2 and Chinese hamster ovary (CHO) cells, monkey COS-1 cells, CV-1, HeLa, mouse L-929, 3T3 cells and BHK cells. The construction and use of some exemplary mammalian vectors and cell lines is well known to those skilled in the art and is discussed in detail in WO 88/00598.

Many strains of yeast cells, known to those skilled in the art, are also available as host cells for expression of the enterokinase activity of the present invention. Yeast cells are especially useful as a host for the PACE pre/pro fusion to mature enterokinase as described above. When expressed using a suitable yeast vector, the fusion is secreted by virtue of the PACE signal peptide, and the PACE pro region is subsequently processed by the endogenous yeast protease KEK2, an enzyme homologous to human PACE which also cleaves after paired basic residues. Additionally, where desired, insect cells may be used as host cells. See, for example, Miller *et al.*, Genetic Engineering 8:277-98(Plenum Press 1986) and references cited therein.

When the enterokinase activity of this invention is expressed in bacterial cells, it may be expressed intracellularly usually without regard to refolding since that is typically unnecessary to obtain the protein in active form, or it may be secreted from bacterial cells in active form, if a secretory leader is included. Where necessary or desired, as when reduced bioactivity is observed, the enterokinase activity product may be refolded by conventional methods such as incubation of protein in urea or guanidine HCl with dithiothreitol or β -mercapto ethanol, followed by dilution to reduce the concentration of these reagents and treatment with oxidizing agents.

For example, *E. coli* cells, genetically engineered to express an enterokinase activity DNA sequence as described herein, are cultured under suitable conditions permitting the production and intracellular accumulation of enterokinase activity protein. The cells are then harvested, *i.e.*, separated from the medium in which they were cultured and from any other materials, and lysed and the desired biologically active enterokinase activity protein is purified from the lysate. Optionally, only minimal purification of the enterokinase activity is required.

The term "biologically active" means a preparation of enterokinase activity that exhibits a detectable level of proteolytic cleavage activity as assayed by conventional methods discussed, *supra*.

Various purification techniques, such as column chromatography (*e.g.*, ion exchange, immunoaffinity, etc.), affinity purification on soybean trypsin inhibitor (STI), pancreatic trypsin inhibitor (PTI) or PABA, gel filtration and reverse phase HPLC, are useful in purifying the desired protein. See, for example, Gospodarowicz *et al.*, J. Cell. Phys 122:323-32(1985), Iwane *et al.*, Biochem. and Biophys. Res. Comm. 146:470-77(1987), Fox *et al.*, J. Biol. Chem. 263:18452-58(1988), EP 0 259 953 published June 4, 1987, and EP 0 237 966 published September 23, 1987.

The enterokinase activity of the invention can be used in a method for cleaving protein having an enterokinase cleavage site, and especially fusion proteins having such a cleavage site engineered into their sequence. The amounts needed are readily determined empirically by one skilled in the art. Indeed, as described herein, recombinant bovine enterokinase catalytic domain is a *superior* reagent for cleavage of fusion proteins when compared to the bovine-derived two-chain form, as it is much more efficient and is not contaminated with trace amounts of other proteolytic proteins which are difficult to remove. As another aspect of the invention, the enterokinase activity of the invention is incorporated as one of the fusion protein partners to yet another protein. As such, with the addition of a minimal amount of exogenous enterokinase activity to the reaction vessel (or by merely concentrating the fusion protein adequately), a minimal amount of cleavage of the fusion protein results in the release of additional enterokinase activity which in turn can catalyze many more proteolytic cleavages of fusion proteins. In this way, large amounts of enterokinase activity can be produced from a fusion protein in an autocatalytic manner. Also provided by the invention is a method for producing proteins from fusion proteins which comprises the steps of:

- (a) growing, in culture, a host cell transformed or transfected with
 - (i) a nucleic acid which encodes enterokinase activity and which upon expression is segregated into the periplasmic space; and
 - (ii) one or more nucleic acids which encode a fusion protein and an enterokinase cleavage site and which, upon expression are segregated to the cytoplasmic space,
- (b) allowing said periplasmic space and said cytoplasmic space to co-mingle thereby,
- (c) allowing said enterokinase activity to cleave said fusion protein, and
- (d) resulting in protein production.

Pharmaceutical compositions containing the homogeneous enterokinase activity of the present invention may be useful as digestive agents. Such pharmaceutical compositions may also contain pharmaceutically acceptable carriers, diluents, fillers, salts, buffers, stabilizers and/or other materials well-known in the art. The term "pharmaceutically acceptable" means a material that does not interfere with the effectiveness of the biological activity of the active ingredient(s) and that is not toxic to the host to which it is administered. The characteristics of the carrier or other material will depend on the route of administration. Administration can be carried out in a variety of conventional ways. Oral

administration is preferred. In such case, the enterokinase activity of the present invention can be enterically coated, the preparation of which is within the skill in the art. In practicing the method of treatment of this invention, a therapeutically effective amount of enterokinase activity is administered. The term "therapeutically effective amount" means the total amount of each active component of the method or composition that is sufficient to show a meaningful benefit, *i.e.*, restoration of digestive function. When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously. The number of applications may vary, depending on the individual and the severity of the digestive disorder. In yet another method of use, it is contemplated that the DNA encoding enterokinase would be useful in gene therapy as a means of correcting digestive disorders due to enterokinase deficiency.

The invention is further described in the following examples, which are intended to illustrate the invention without limiting its scope. Example 1 describes the cloning of a 26 bp bovine enterokinase gene fragment. Additional protein sequencing of bovine enterokinase is described in Example 2. The amplification and cloning of a gene fragment adjacent to the Example 1 fragment is the subject of Example 3. Example 4 relates to the cloning of the enterokinase catalytic chain. A comparison of the different cDNA clones, as well as a partial coding sequence for the non-catalytic (heavy) chain is set forth in Example 5. Example 6 describes the isolation of additional enterokinase coding sequence including additional heavy chain sequence. Example 7 describes the use of the bovine enterokinase sequence to clone other mammalian enterokinase genes. Example 8 describes the expression of a gene encoding the catalytic domain of bovine enterokinase in both a procaryotic cell system as well as in a eukaryotic cell system. Example 9 relates to the co-expression of fusion proteins and to the production of active enterokinase. Example 10 relates to the use of enterokinase as a therapeutic agent in the treatment of certain digestive disorders.

EXAMPLE 1

CLONING OF A BOVINE
ENTEROKINASE GENE FRAGMENT

A purported N-terminal 27 amino acid sequence of the catalytic (light) chain of bovine enterokinase was provided by Albert Light of Purdue University and was later published in Light *et al.*, *supra*. As discussed in greater detail, *infra.*, this sequence was incorrect. Because of the error, the tyrosine reported at position 8 was used in designing probes and primers due to its low degeneracy (only two possible codons encode tyrosine). However, the actual residue at position 8 is in fact arginine, with six possible codons. This (erroneous) sequence is as follows:

SEQ ID NO:3

```

      1             10             20             27
      I V G G S D S Y E G A W P W V V A L - - - Y F D D Q - Q V C G

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The "provided" sequence was backtranslated into all possible DNA codons which it could encode, and was used to design pools of oligonucleotide primers 17 base pairs in length with 5' extensions to encode restriction endonuclease cleavage sites to be used as primers in PCR reactions [Saiki *et al.*, Science 230:1350-1354(1985); Mullis *et al.*, Cold Spring Harbor Symposia on Quantitative Biology, Vol. LI:263-273(1986)]. The design of these oligonucleotide pools was critical to the potential success of the endeavor. Comparison of this N-terminal protein sequence to previously identified sequences in the databases revealed significant homology to a large number of mammalian pancreatic and serum serine proteases. To prevent unwanted amplification of DNA sequences encoding these "unwanted" proteins, the PCR primer pools were designed to intentionally avoid these highly homologous regions. However, the competing requirement of spacing the sequences to which the primer pools anneal as far apart as possible, was taken into account to maximize the amount of exact enterokinase sequence generated for the amplification to provide useful information.

Two degenerate oligonucleotide pools, which together contained all possible codons for the N-terminal amino acid sequence: IVGGSD (amino acids 1-6) SEQ ID NO:8, were synthesized. These two pools differed only in the codons used for the serine residue in the protein sequence and were used independently as a means of decreasing the degeneracy of each pool:

SEQ ID NO:4

PRIMER 1A 5' CTCGAATTCATHGTNGGNGGNTCNGA 3' 768x

and

SEQ ID NO:5

PRIMER 1B 5' CTCGAATTCATHGTNGGNGGNAGYGA 3' 384x

As used herein, the symbol "H" refers to equal proportions of nucleotides C, T, and A. The symbol

"Y" refers to equal proportions of nucleotides C and T. "R" refers to equal proportions of either A or G at that position. The symbol "N" refers to equal proportions of the four nucleotides G, A, T, and C. Each of these pools had a 5' extension which contained an EcoRI site which is shown in **bold print**.

5 Another pool of oligonucleotides was synthesized which contained the reverse complement of all possible codons for the most C-terminal known sequence:

SEQ ID NO:6

DQQVCG (amino acids 22-27 of SEQ ID NO:3).

This pool contained a common 5' extension shown in **bold** which contained a HindIII site:

10

SEQ ID NO:7

PRIMER 2 5' **TCCAAGCTTCCRCANACYTGYTGRTC** 3' 64x

15 The DNA products from the first series of amplification reactions were used as the template for a second series of amplifications primed by oligonucleotide pools which are complementary to the inferred DNA coding sequence of amino acids "interior" to the first set in the linear sequence. Thus, a pool of 17 base pair oligonucleotides complementary to all possible codons for the sequence:

SEQ ID NO:8

20 YEGAWP (which corresponds to amino acids 8-13 of SEQ ID NO:3, including the incorrect assignment of Y at position 8) was synthesized:

SEQ ID NO:9

PRIMER 3 5' TAYGARGGNGCNTGGCC 3' 64x

25 This pool of primers was then combined in the second PCR reaction with another pool comprising the reverse complement of all possible codons for the sequence:

SEQ ID NO:10

FDDQQV (corresponds to amino acids 20-25 of SEQ ID NO:3)

30

SEQ ID NO:11

PRIMER 4 5' TCCAAGCTTACYTGYTGRTCTCRAA 3' 32x

This pool has partial overlap with the 3' pool used in the first series of amplifications, and contains a 5' extension (in **bold**) which includes a HindIII site.

5 Bovine genomic DNA (0.9 mg/ml in dH₂O) was boiled for 5 minutes to denature and was then immediately placed on ice. Reaction conditions for each 50 μ l amplification reaction were: 2 μ g heat denatured bovine genomic DNA, 10 mM Tris-Hcl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 1.0 μ M of each oligonucleotide pool, 200 μ M of each dNTP, and 1 unit of Amplitaq DNA polymerase (Perkin-Elmer Cetus). Forty amplification cycles were run under the following conditions: cycle 1 =
10 94°C, 3 minutes/40°C, 1 minute/72°C, 1 minute. Cycles 2-40 = 94°C, 1 minute/40°C, 1 minute/72°C, 1 minute. The first round of 40 cycles utilized either primer pools 1A and 2 or pools 1B and 2. After 40 cycles of amplification, 0.5 μ l of this reaction was used as template for a second 35 cycles of PCR using primer pools 3 and 4. The conditions for this round of 35 PCR cycles were 94°C, 1 minute/35°C, 2 minutes/72°C, 2 minutes. Reaction components were the same as the first round
15 except for the DNA template. The DNA template in the second round was the product of the previous round.

 PCR products obtained as described above, were run on 5% acrylamide preparative gels, and bands were stained with 0.5 μ g/ml ethidium bromide, excised from the gel and electroeluted. DNA manipulations and ligations were performed using standard techniques [Sambrook *et al.*, in "Molecular Cloning, a Laboratory Manual," second edition, Cold Spring Harbor Laboratory Press. (1989)]. PCR products were first treated with Klenow fragment of DNA polymerase I in the presence of all four deoxynucleotide triphosphates, then digested with HindIII (New England Biolabs) and subcloned into a pUC19 [Norrander *et al.*, Gene 26:101-106(1983)] HincII-HindIII vector. Transformants were identified which contained plasmids with an apparent insert of approximately 72 bp. These plasmids were isolated
25 and their inserts were sequenced using the Sequenase kit (United States Biochemical) and a sequencing primer which anneals to pUC19. The DNA sequence of the inserts was then translated to reveal an open reading frame which corresponded exactly to the amino acid sequence predicted by the known protein sequence (WVVALY, amino acids 14-19 of SEQ ID NO:3). Due to the possibility of mismatch tolerance during primer annealing, only the sequence between the two PCR primers could be assumed
30 to be correct. However, it was assumed that the proper serine codon was in primer pool 1B (AGY), as the other pool (1A) failed to yield a specific product; this was determined by Southern blot of product from the first 40 cycles, probed with pool designated SEQ ID NO:9. Also, since the "wobble" position of the codon for Pro¹³ was determined to be a thymidine, and there is only one possible codon for the adjacent Trp¹², 5 additional bases were also assumed to be fairly certain. When the first two invariant
35 bases of the codon for Phe²⁰ are included, 26 contiguous base pairs of coding sequence for nine amino

acids of the enterokinase catalytic chain (amino acids Trp¹² to Phe²⁰) had been determined with a fair degree of certainty. This sequence is nucleotides 1724 to 1749 of SEQ ID NO:1.

EXAMPLE 2

5 PROTEIN SEQUENCING OF BOVINE ENTEROKINASE

The exact DNA sequence (26 bp) of amino acids 12-20 of mature bovine enterokinase light chain was not sufficient to allow cDNA isolation by a hybridization approach. Accordingly, additional adjacent protein sequence was sought.

10 Bovine enterokinase (EK-2 grade) was purchased from Biozyme. The enzyme was greater than 99% impure, thus the enzyme was further purified using porcine pancreatic trypsin inhibitor (Sigma) coupled to activated Sepharose CL-4B (Sigma) [Liepnieks *et al.*, J. Biol. Chem. 254:1677-1683(1979)]. The resulting enzyme was reduced and alkylated to separate the heavy chain from the light chain and run on a preparative acrylamide gel. The proteins were electroblotted from the gel onto a Problot membrane (Applied Biosystems, Inc.), and the catalytic chain of M_r 42,000 daltons was excised from
15 the membrane after staining and was sequenced using an Applied Biosystems Model 470A pulse liquid sequencer. The sequence for the first 30 amino acids was determined and is:

SEQ ID NO:12

20 1 10 20 30
 I V G G S D S R E G A W P W V V A L Y F D D Q Q V C G A S L

Of particular note is the observation that the amino acid residue in the 8th position was determined to be an arginine, in contrast to the tyrosine incorrectly reported by Light *et al.*, *supra*. This is a crucial area of the sequence for designing PCR primers due to its reported low degeneracy.

25 Two additional bands were observed upon electoblotting. The expected heavy chain band at M_r 150,000 daltons and another band at M_r 90,000 daltons were excised from the Problot membrane, treated individually with trypsin, and the resulting fragments separated on reverse phase. Well-separated peaks were collected and sequenced.

30 The reduced and alkylated bovine enzyme was also run on a C4 reverse phase column (Vydac) to separate the non-catalytic (heavy) chain from the catalytic (light) chain. The peak corresponding to the catalytic chain was treated with TPCK-trypsin (Worthington). The resulting tryptic peptides were separated on C18 reverse phase HPLC (Vydac). Individual peaks were subjected to sequence analysis on the protein sequencer. The results are presented in Example 3.

EXAMPLE 3

AMPLIFICATION AND CLONING
OF AN ADJACENT GENE FRAGMENT

5 Tryptic digestion and chromatographic separation and isolation of individual peptide fragments of enterokinase catalytic chain, followed by subsequent sequencing of each resulting peptide, resulted in the following sequences:

SEQ ID NO:13

E G A W P W V V A L Y F D D Q Q V C G A S L V S

10 SEQ ID NO:14

DWLVSAAHCVYGR

SEQ ID NO:15

FTEWIQSFLH

15

SEQ ID NO:16

ICSIAGWGALIYQGSTADVLQEA

SEQ ID NO:17

20 WLLAGVTSFGYQCALPN(N?)PGVYA

SEQ ID NO:18

NMEPSK

25 SEQ ID NO:13 is a 24 residue peptide that partially overlapped with the N-terminal sequence as determined in Example 2. These peptide sequences were used to search protein sequence databases for homology. The protein which displayed the highest degree of sequence homology to the N-terminal peptide sequence of enterokinase was an inferred amino acid sequence from a human liver cDNA clone, named hepsin [Leytus *et al.*, Biochemistry 27:1067-1074(1988)]. Using the hepsin sequence as a guide, another enterokinase catalytic chain tryptic peptide (SEQ ID NO:14) appeared it might be contiguous
30 with the N-terminal/overlapping tryptic sequence already identified. This peptide contained a sequence highly homologous to the histidine region of the "catalytic triad" which is characteristic of serine proteases. Oligonucleotide pools which were complementary to the reverse complement of the backtranslated amino acid sequence for a region of this peptide, AHCYVY, were synthesized. These
35 oligos also contained a 5' extension (shown in bold) which encodes a BamHI site:

SEQ ID NO:19

PRIMER 5 5' CGCGGATCCCCRTANACRCARTGNGC 3' 128x

- This pool was used for genomic DNA PCR in combination with the other oligonucleotide pools and also with oligonucleotides containing the 19 base pairs (and the reverse complement) of exact enterokinase DNA sequence (nucleotides 1729-1747) from the first PCR clone in Example 1, along with 5' restriction site extensions, as shown below:

SEQ ID NO:20

CCGGAATTCTTGGGTCGTTGCTCTGTAT

10

SEQ ID NO:21

CGCGGATCCATACAGAGCAACGACCCAA

- A nested approach was again utilized on bovine genomic DNA. The following combinations proved fruitful:

- 1) SEQ ID NO:5 + 7, 40 cycles followed by SEQ ID NO:5 + 21, 35 cycles
- 2) SEQ ID NO:5 + 19, 40 cycles followed by SEQ ID NO:20 + 19, 35 cycles

- Combination 1, after subcloning and sequencing, yielded 21 bp of sequence (5 bp of which had previously been determined) which translated to Asp⁶ to Pro¹³ of the enterokinase light chain peptide sequence SEQ ID NO:12, and the inferred remaining two bases of the Asp⁶ codon to total 23 bp. This DNA sequence corresponds to nucleotides 1706 to 1728 of SEQ ID NO:1 and confirmed that residue #8 was indeed an arginine and not a tyrosine as had been incorrectly reported by Light *et al.*, *supra*.

- Likewise, combination 2 yielded 60 bp of sequence (2 bp of which had previously been determined) which translated to Phe²⁰ to Ala⁴⁰ of the enterokinase light chain N-terminal protein sequence (SEQ ID NO:12) and the overlapping tryptic peptide sequence (SEQ ID NO:13) and showed that adjacent peptide SEQ ID NO:14 begins with residue 34. The residue at position 33, which was undetermined from protein sequencing but was presumed to be a basic residue responsible for the tryptic hydrolysis, was determined to be an arginine from the coding sequence. When the sequences for all three PCR products were combined, a total of 104 bp of exact, contiguous coding sequence had been determined for Asp⁶ to Ala⁴⁰ (and the first two bases of the codon for Ala⁴¹) of the catalytic chain of bovine enterokinase. With this sequence information in hand, only now was it possible to attempt to clone the catalytic domain of enterokinase with any reasonable chance of success.

EXAMPLE 4

CLONING OF ENTEROKINASE CATALYTIC CHAIN

Two separate bovine small intestine cDNA libraries were used for the cloning of the gene for the enterokinase catalytic domain. PCR was performed on λ libraries of bovine liver and small intestine cDNA's using exact primers designed to this newly determined nucleotide sequence as described *supra*.
5 cDNA from bovine liver gave a very weak product implying that the abundance in the library was very low, while the small intestine library yielded much more specific product. Thus bovine small intestine was chosen as a possible mRNA source. The first cDNA library was a λ gt10 library which was purchased from Clontech. The second cDNA library, referred to as the Lambda Zap library,
10 was prepared as follows. Bovine duodenal tissue was obtained and mRNA was prepared from a portion of the tissue using the guanidinium extraction method [Chirgwin *et al.*, Biochemistry 18:5294(1979)]. Oligo (dT)-primed cDNA was synthesized using standard techniques [Sambrook *et al.*, *supra*]. Synthetic NotI/EcoRI adapters (Invitrogen) were ligated to the resulting cDNA, which was then ligated into Lambda Zap II Eco RI arms (Invitrogen).

15 Recombinant phage from either cDNA library were hybridized, in duplicate, to two separate oligonucleotides whose sequences were complementary to the enterokinase DNA sequence determined from the subcloned PCR fragments of Example 3. The first oligonucleotide was 21 bases in length and comprised the plus strand of the coding sequence for Asp⁶ to Trp¹². The second oligonucleotide was 20 bases in length and comprised the minus strand of the coding sequence for residues Asp³⁵ to Ala⁴¹.

20 The oligonucleotides were labelled using [³²P]- γ ATP and polynucleotide kinase [Sambrook *et al.*, *supra*]. Hybridizations were performed as described [Sambrook *et al.*, *supra*] using the following conditions: 6x SSC, 0.5% SDS, 5x Denhardt's solution, 10mM Na₂EDTA, 100 μ g/ml yeast RNA, and 0.1 pmole/ml labelled oligonucleotide. After hybridization for 16 hours at 60°C, filters were washed in 2xSSC, 0.1% SDS at room temperature 4 times for 15 minutes each time.

25 A single plaque containing sequences which hybridized to both of the oligonucleotide probes was isolated from 1x10⁶ recombinant phage from the Clontech library. The sequence of the insert (called clone #3e) in this recombinant phage was 769bp. The insert contained a long open reading frame which encoded several of the tryptic peptides previously sequenced: SEQ ID NO:13,14 17, as well as a portion of SEQ ID NO:16. The reading frame continued past the 3' end of the insert, suggesting that the clone
30 was incomplete. In addition, the reading frame contained the IVGG- N-terminus predicted from the protein sequencing data. The reading frame remained open in the 5' direction for another 26 codons before terminating.

From the Lambda Zap II bovine small intestine cDNA library, 5 X 10⁵ phage were screened by hybridization with the same two oligonucleotides as above. Only two recombinant phage were isolated
35 which contained enterokinase-specific sequence complementary to the oligonucleotide probes. One of

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these (called clone #11) was 1494 base pairs long and contained all of the light chain coding sequence present in the cDNA clone from the Clontech library but differed 5' of the coding sequence for light chain. It also contained the remaining 3' coding sequence and almost 80 bases of 3' untranslated sequence. This clone also contained a significant extension of the open reading frame preceding the N-terminal IVGG of the mature catalytic chain, extending 266 codons and remaining open at the 5' limit of this clone (to nucleotide 893 of SEQ ID NO:1), which differed with clone 3e.

The second phage had an insert (called clone #22) which was considerably smaller, only 531 bp (SEQ ID NO:1, nucleotides 1553-2068), the sequence of which was fully contained within the first clone. Of interest, however, were the final 21 codons of the open reading frame contained on clone #22 which were not present in either of the other two cDNA clones (SEQ ID NO:1, nucleotides 2006-2101). Thus, it was unclear where this sequence fit in and/or whether it was merely a cloning artifact.

EXAMPLE 5

COMPARISON OF DIFFERENT cDNA CLONES

Comparison of the Clontech library clone #3e with clone #11 revealed that the two sequences diverge at almost exactly the point at which the 5' open reading frame of clone #3e terminates. Examination of the DNA sequence surrounding this point reveals a potential mRNA splice site [Padgett *et al.*, Ann. Rev. Biochem. 55:1119-1150(1986)], and leaves open the possibility that clone #3e contains an unspliced intron which interrupts the open reading frame (ORF). Further support for this possibility comes from the identification of a tryptic sequence:

SEQ ID NO:22

LVTQEVSPK

isolated from the 150,000 dalton protein fragment which matches the ORF sequence immediately preceding the IVGG N-terminal sequence of the mature catalytic chain in clone #11. This tryptic sequence is interrupted by divergent sequence in clone #3e. In addition, two other tryptic peptides:

SEQ ID NO:23

A-FTTGYGLGIPEP and

SEQ ID NO:24

LF-GTTDSSGLVQF

isolated from the 150,000 dalton enterokinase protein band match two regions of the translated ORF of clone #11 upstream of the catalytic chain coding sequence. Therefore, this upstream ORF apparently represents the coding sequence for the non-catalytic (heavy) chain, which is believed to be generated

from a single proteolytic cleavage immediately prior to the mature catalytic chain N-terminal sequence to separate the mature catalytic chain from the non-catalytic chain.

EXAMPLE 6

5 ISOLATION OF ADDITIONAL ENTEROKINASE CODING SEQUENCE

Nested oligonucleotide primers were synthesized which were complementary to the lambda DNA sequence adjacent to the cloning site for the cDNA insertions. These primers are shown as Lambda Primers below. In addition, primers were designed which are complementary to the plus strand of the most 5' region of the enterokinase coding sequence as described, *supra*. These primers are shown as EK Primers below. The innermost primers were designed to contain a 5' extension (shown in bold) to encode a restriction endonuclease cleavage site.

Lambda Primers

15 SEQ ID NO:25

5' CTATAGACTGCTGGGTAGTCCCC 3' OUTER

SEQ ID NO:26

5' ATAAGAATGCGGCCGCAAGTTCAGCCTGGTTAAGTCCAAGC 3' INNER

20 EK Primers

SEQ ID NO:27

5' CCAAATACAGAAAGCCTGATTAGGG 3' OUTER

25 SEQ ID NO:28

5' GTAGGTGCGACCGTGAATGTTGTATTGGCTCCC 3' INNER

Nested PCR was then performed as follows: each 100 μ l reaction contained 1×10^8 recombinant phage from the Clontech bovine small intestine lambda gt10 cDNA library, 1 μ mole of each outer primer, 200 μ M dNTPs, and 1 unit of Amplitaq (Perkin-Elmer Cetus) in a final concentration of 10 mM Tris-HCl pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$. Thirty-five cycles were performed under the following conditions: 94°C, 1 minute; 65°C, 2 minutes; 72°C, 2 minutes. Five microliters were removed from this reaction and used as template for another 35 cycles utilizing the inner primers and the same reaction conditions. The products from this reaction were then run on a 1% polyacrylamide gel, stained in a solution of 0.5 μ g/ml ethidium bromide and visualized under UV light. The resulting bands were excised, electroeluted, and digested with NotI and SalI prior to subcloning into a pBluescript SK+ (Stratagene) NotI/SalI vector. The resulting subclones were sequenced, and additional DNA sequence

encoding another 116 amino acids of enterokinase heavy chain was determined. This new protein sequence contained regions corresponding to two additional tryptic peptides which had been isolated from the 150 Kd protein band, (LS)INISSDQNMEK [SEQ ID NO:29] and VSFYGFK [SEQ ID NO:30], and another from the 90 Kd protein band QKEGNYGQNWNYGQVTLNET [SEQ ID NO:31]. In addition, two separate N-termini were sequenced from the 90 Kd band, and both are identified in this sequence: (VGLLTLP...)[SEQ ID NO:32] and (TIFQK...)[SEQ ID NO:33]. Thus, the 90 Kd protein band seen in reduced and alkylated bovine-derived enzyme may be a proteolyzed form of the heavy chain which runs at an apparent 150 Kd on these gels. These two N-termini both follow a basic residue, indicating that a trypsin-like enzyme may be responsible for proteolysis of the intact heavy to this smaller (90kD) form.

Using the technique described above, the complete heavy chain sequence is obtained by repeating this method each time additional 5' coding sequence is identified. New nested PCR primers are designed to the most 5' sequence, and nested PCR with 1×10^8 recombinant phage from a bovine small intestine λ cDNA library is performed. Additional 5' enterokinase heavy chain coding sequence is thus amplified, subcloned, sequenced and the procedure repeated until the entire coding sequence has been identified and isolated.

EXAMPLE 7

USE OF THE BOVINE ENTEROKINASE DNA SEQUENCE TO CLONE OTHER MAMMALIAN ENTEROKINASE GENES

The determination of the DNA sequence of bovine enterokinase directly enables isolation and sequencing of the genes for the equivalent enzymes from numerous other mammalian species. The subject invention determined that the enterokinase gene is expressed in the duodenum, thereby eliminating the uncertainty of tissue sources for enterokinase mRNA regardless of species. This information provides assurance that cDNA made from duodenal mRNA will contain the enterokinase gene. Thus, expression libraries containing cDNA made from duodenal mRNA from virtually any mammalian species can be screened using antibodies to the particular enterokinase protein which is sought. Polyclonal antibodies to the bovine enzyme are useful for identification of enterokinase clones from expression libraries made from other species.

In addition, the cDNA sequence for the bovine enzyme can be used directly as a hybridization probe for other mammalian enterokinase genes, or can be used to design oligonucleotide probes useful for cloning enterokinase genes from cDNA or genomic libraries made from mRNA or genomic DNA of other species. It is reasonable to assume that the enterokinase catalytic domain protein sequence will be highly conserved among species due to the fact that it now appears that there is nearly absolute conservation of the substrate recognition sequence. Using the entire bovine enterokinase catalytic domain

as a hybridization probe to screen cDNA or genomic libraries of other species at reduced stringency allows isolation of the desired enterokinase gene. Alternatively, oligonucleotides which encompass the DNA sequence encoding the regions surrounding the "catalytic triad", *i.e.*, His⁴¹, Asp⁹², and Ser¹⁸⁷ are likely to be most highly conserved and most useful for cross-species hybridization.

5

EXAMPLE 8

EXPRESSION OF THE GENE ENCODING THE CATALYTIC DOMAIN OF BOVINE ENTEROKINASE

10 A. CHO Cell Expression

1. PACE

The DNA sequence encoding the catalytic domain (nucleotides 1691 to 2398) of bovine enterokinase, was fused in-frame to the 3' end of the DNA encoding the signal peptide and pro- region of the human PACE gene. PACE is a mammalian serine protease which cleaves after dibasic residues, and is responsible for propeptide processing of a number of secreted proteins. (Wise *et al.*, Proc. Natl. Acad. Sci. USA 87:9378-9382(1990). When expressed in CHO cells, this sequence was translated to produce a chimeric protein which was secreted with subsequent signal peptide processing to yield pro-enterokinase. The PACE pro-peptide contains a sequence (-Arg-Thr-Lys-Arg-) at the C-terminal junction with mature enterokinase sequence; this is the cleavage site for the PACE enzyme. CHO cells also produce endogenous levels of PACE. During secretion of the PACE pro/enterokinase light chain, host PACE cleaved the pro-peptide from the N-terminus of the enterokinase, resulting in secretion of mature enterokinase catalytic domain to the conditioned media. Immunoprecipitation experiments using rabbit polyclonal antisera raised against bovine-derived enterokinase revealed a 42 Kd product was secreted into the conditioned media.

25 This conditioned media contained cleaving activity toward the fluorogenic enterokinase substrate Gly-(Asp₄)-Lys-βNA (Bachem Bioscience)(corresponding to approximately 50-500 ng/ml depending on the cell line). This activity was inhibited by the addition of either soybean trypsin inhibitor (STI, Sigma) or bovine pancreatic trypsin inhibitor (BPTI, Sigma). It has been reported that the bovine holoenzyme, *i.e.*, having both heavy and light chains, is inhibited by only BPTI and not STI, while the partially reduced and alkylated light chain is inhibited by both [Light *et al.*, J. Biol. Chem. 259:13195-13198(1984)]. In addition, incubation of this conditioned media with a partially purified fusion protein of *E. coli* thioredoxin/human IL-11 which contains an interdomain spacer consisting of the enterokinase cleavage sequence (-Gly-Ser-Gly-Ser-Gly-[Asp₄]-Lys-Asn-) resulted in total and specific cleavage of this fusion protein into its two component domains (thioredoxin and IL-11), with cleavage occurring between the Lys and Asn residues in the spacer sequence. In addition, this CHO-produced recombinant enterokinase catalytic domain was capable of specifically cleaving other fusion proteins containing this

35

same spacer, for instance an *E. coli* thioredoxin/human MIP-1 α fusion and an *E. coli* thioredoxin/human MIF fusion, into their component parts. This cleavage was confirmed by SDS-PAGE analysis of the cleavage products.

The relative molar activities were as follows:

EFFICIENCY OF CLEAVAGE

	Substrate	Holoenzyme	CHO Produced Light Chain
10	Gly-(Asp4)-Lys- β NA	1	1
	Trypsinogen	100	1
	Trx/IL-11	1	25

Quite surprisingly, the CHO-produced light chain is 25 times more effective than bovine-derived holoenzyme when used to cleave a thioredoxin/IL-11 fusion protein containing an enterokinase cleavage site between the two protein domains. This dramatic difference is duplicated on the other fusion proteins listed above. In addition, secondary proteolysis due to contaminating serine proteases (e.g. trypsin and chymotrypsin) which co-purify with bovine-derived holoenzyme is absent with the recombinant single chain form. As such, recombinant single chain enterokinase is a superior reagent for fusion protein cleavage.

2. Modified PACE

This expression system has been improved by co-expression of a modified version of the PACE gene which has had the transmembrane domain deleted. Rehemtulla *et al.*, *Blood* 79:2349 (1992). This overexpressed and secreted PACE efficiently processes the PACE pre/pro-enterokinase and allows greater processing capability for enterokinase overexpression, as endogenous PACE levels in CHO cells are low and incapable of processing highly expressed pro-enterokinase. Thus, at high expression levels, endogenous PACE activity becomes limiting with some enterokinase remaining unprocessed and resulting in some inactive material. Increasing soluble PACE levels allow for the accumulation of high levels of properly processed, active enterokinase in the conditioned media.

3. Trypsinogen

Constructs were also prepared which fused the DNA encoding the pre/pro region of bovine anionic trypsinogen [Le Heurou *et al.*, *Eur. J. Biochem.* 193:767-773(1990)] in-frame to the DNA sequence encoding the mature enterokinase catalytic domain. The pro region of trypsinogen contains an enterokinase cleavage site (Asp₁-Lys) as it is the natural substrate of enterokinase, and this construct was designed to produce secreted enterokinase "zymogen" with the trypsinogen propeptide attached to its N-terminus which could then be activated by addition of enterokinase to initiate autocatalytic processing. Expression of this construct in CHO cells resulted in mostly intracellular accumulation;

however, the small amount of material secreted gave undetectable levels of activity in a fluorogenic enterokinase peptide assay. In addition, activity was not stimulated by the addition of enterokinase to the proprotein. It appears that this chimeric protein is not capable of forming an active species. It is speculated that the light chain benefits from translational fusion with a large protein domain (analogous to heavy chain) which is post-translationally removed to allow the active conformation of light chain to form. The PACE pro-peptide functioned effectively in this capacity.

B. Expression In *E. Coli*

In an effort to increase solubility and produce enterokinase with an authentic N-terminus, the coding sequence for the catalytic chain was fused in-frame to the 3' end of the *E. coli* thioredoxin gene [Lunn *et al.*, J. Biol. Chem. 259:10469-10474(1984)] with a spacer which encodes an enterokinase cleavage site (-Gly-Ser-Gly-Ser-Gly-[Asp₄]-Lys). This construct is under the transcriptional control of the lambda pL promoter [Shimatake *et al.*, *supra.*] on a multicopy plasmid, and directs the cytoplasmic expression of a thioredoxin/enterokinase catalytic domain fusion protein. A portion of the expressed fusion protein is soluble when expressed at 17°C, and full solubility can be achieved by lysing the cells in the presence of low levels of urea (*e.g.*, 3 M). This fusion protein can be purified from cell lysates and cleaved with enterokinase to generate active enterokinase. The intent of this construct is to allow autocatalytic processing of the fusion protein, *i.e.*, cleavage is begun by a small amount of active enterokinase (either holoenzyme or catalytic chain), and as active catalytic chain is released from its fusion partner it can then continue to cleave remaining fusion protein in the reaction. At least partial purification of the fusion protein is necessary to eliminate inhibitor(s) of enterokinase present in *E. coli* cell lysates. Active light chain, specifically inhibited by STI, is produced.

Alternatively, other fusion partners may also be employed. For instance, the *E. coli* maltose-binding protein, a secreted protein which has been described as a competent fusion partner (Maina *et al.*, Gene 74:365 [1988]) has been used with success. We anticipate that other fusion strategies may also serve to allow proper folding and provide a means to produce authentic, active enterokinase light chain.

C. Expression in *Saccharomyces cerevisiae*

1. PACE

The expression construct described for use in CHO cells utilizing the PACE pre/pro sequence fused to the 5' end of the coding sequence for mature bovine enterokinase catalytic chain can also be used for enterokinase secretion from *Saccharomyces cerevisiae*. This yeast has been shown to produce an enzyme called Kex2 [Julius *et al.*, Cell 37:1075 (1984)] which cleaves on the C-terminal side of dibasic residues, similarly to PACE. Co-expression of the yeast *kex2* gene with the PACE pre/pro-bovine enterokinase light chain construct in COS cells results in complete processing of the PACE

pre/pro sequence to yield a product which is immunoprecipitable with bovine enterokinase antisera and co-migrates with PACE-processed enterokinase light chain after separation of the products on SDS-PAGE. Thus, yeast Kex2 recognized and cleaved the PACE cleavage site in the PACE pro-sequence to produce mature enterokinase.

5 The coding sequence for this chimeric construct (mammalian PACE secretory leader and propeptide sequence followed by the mature bovine enterokinase light chain sequence) was inserted into a yeast expression vector to produce and secrete the fusion protein. The host Kex2 protein is expected to cleave off the PACE pro-peptide following the Arg-Thr-Lys-Arg sequence, resulting in secretion of properly processed mature enterokinase light chain. The Kex2 protein may be co-expressed to increase
10 processing activity if needed. Such over-expression of Kex2 may be accomplished with either the native protein or with a soluble derivative lacking the C-terminal transmembrane domain as described by Brenner *et. al.*, *supra.*. This form of Kex2 is analogous to the soluble PACE co-expressed with the PACE pre/pro-bovine enterokinase light chain used in the CHO cell expression. Alternatively, mammalian PACE can be co-expressed in yeast to accentuate pro-peptide processing of the chimeric
15 enterokinase construct either in the presence or absence of host endogenous levels of Kex2.

2. α -Factor

Alternatively, the coding sequence for mature bovine enterokinase light chain can be fused to the coding sequence for the secretory leader and pro-peptide of, for instance, the α -factor protein from
20 *S. cerevisiae*, a protein which is normally secreted and subsequently processed by Kex2 [Julius *et. al.*, *Cell* 32:839 (1983)]. This construct is expected to produce material similar to the other construct described above; that is, properly processed and active enterokinase light chain which accumulates in the culture media in active form.

EXAMPLE 9

CO-EXPRESSION OF FUSION PROTEINS AND ACTIVE ENTEROKINASE

25 A configuration advantageous in some situations co-expresses active enterokinase along with a fusion protein which is to be subsequently cleaved. The fusion can be segregated by cell compartmentalization during cell growth and fusion protein synthesis, thereby allowing the desirable effects of fusion proteins (*e.g.*, stabilization, solubility) to remain. Then, upon cell lysis, the active enterokinase is allowed to mix (co-mingle) with the expressed fusion protein and cleave it, thereby simplifying the downstream processing of the fusion protein. One method for accomplishing this is to
30 secrete active enterokinase into the periplasmic space of *E. coli*, while producing a fusion protein in the cytoplasm. Other methods can be equally suitable, for instance co-secretion of enterokinase and a fusion
35 protein in CHO cells, analogous to the co-secretion of PACE and the PACE pro/enterokinase fusion

employed for CHO production of active enterokinase as described, *supra*. Another method is co-expression of an enterokinase fusion protein (e.g., Trx/enterokinase light chain with an enterokinase cleavage site between them) and a fusion protein containing a desired protein product, also with an enterokinase site between the domains. The enterokinase is expected to remain inactive until purified and until concentrated to the point where autocatalysis occurs, whereupon the co-purified desired fusion protein will also be processed.

EXAMPLE 10

USE OF ENTEROKINASE AS A THERAPEUTIC AGENT

A condition exists in humans whereby the ability to digest protein is severely impaired [Hadorn *et al.*, *Lancet* 1:812-813(1969); Tarlow *et al.*, *Arch. Dis. Child.* 45:651-655(1970)]. Studies on these patients have revealed that they are deficient in the production of enterokinase (enteropeptidase), which is necessary for the conversion of trypsinogen to trypsin which in turn activates the numerous pancreatic zymogens responsible for digestion to occur. Duodenal juice from these patients cannot activate trypsinogen *in vitro*, but addition of purified enterokinase to this duodenal juice results in activation of proteolytic enzymes, suggesting that the inactive zymogens are present and able to be activated [Hadorn *et al.*, *supra*]. This condition has been treated in the past with pancreatic extracts.

A recombinant enterokinase may be used as a therapy for this condition. When formulated to allow oral administration, the enzyme enters the duodenum where it encounters the inactive pancreatic zymogens entering from the pancreatic duct. There it activates trypsinogen which in turn activates the other zymogens, and proper digestion proceeds. The human form of enterokinase gene may also be useful in gene therapy to correct this condition.

The foregoing illustrative examples relate to the isolation and characterization of nucleic acid sequences encoding enterokinase activity, as well as the corresponding transcription and translation thereof to yield the corresponding proteins and polypeptides. Also described are the uses of these proteins either as a heavy and light chain together, or a light chain alone.

While the present invention has been described in terms of specific methods and compositions, it is understood that variations and modifications will occur to those skilled in the art upon consideration of the present invention.

Numerous modifications and variations in the invention as described in the above illustrative examples are expected to occur to those skilled in the art and consequently only such limitations as appear in the appended claims be placed thereon. Accordingly, it is intended in the appended claims to cover all such equivalent variations which come within the scope of the invention as claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: GENETICS INSTITUTE, INC.
(B) STREET: 87 CambridgePark Drive
(C) CITY: Cambridge
(D) STATE: Massachusetts
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 02140
(G) TELEPHONE: (617) 876-1170
(H) TELEFAX: (617) 876-5851

(ii) TITLE OF INVENTION: CLONING OF ENTEROKINASE AND METHOD OF USE

(iii) NUMBER OF SEQUENCES: 33

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2581 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGGAGCTTGT GATGGAAGAT TTTTGTGAC TGGATCTTCT GGGTCCTTTG AGGCTCTGCA	60
TTATCCCAAG CCTTCTAATA ATACAAGCGC TGTTTGTCCG TGGATTATAC GTGTAAACCA	120
AGGACTTTCC ATTCAACTGA ACTTCGATTA TTTTAATACA TATTATGCAG ATGTATTAAA	180
TATTTATGAA GGAATGGGTT CAAGCAAGAT TTTAAGAGCT TCTCTCTGGT CAAATAATCC	240
TGGCATAATT AGGATTTTTT CCAATCAAGT TACTGCCACT TTTCTTATAC AGTCTGATGA	300
AAGTGATTAT ATTGGCTTCA AAGTAACATA CACTGCATTT AACAGCAAAG AGCTTAATAA	360
TTATGAGAAA ATCAACTGTA ATTTTGAAGA TGGCTTCTGT TTCTGGATCC AGGATCTAAA	420
TGATGACAAT GAGTGGGAAA GGA CTCAGG AAGCACCTTT CCTCCATCTA CTGGACCAAC	480
TTTTGACCAC ACTTTTGGCA ATGAGTCAGG ATTTTACATT TCCACCCCAA CTGGACCAGG	540
AGGAAGACGA GAAAGAGTAG GACTTTTAAC TCTCCCTTTA GATCCCACTC CTGAACAAGC	600
CTGCCTTAGT TTCTGGTATT ATATGTATGG TGAAAATGTT TACAACTAA GCATTAATAT	660
CAGCAGTGAC CAAAACATGG AGAAGACAAT TTTCCAAAAA GAAGGAAATT ATGGACAAAA	720
TTGGAAGTAT GGACAAGTAA CATTAAATGA AACAGTGGAA TTTAAGGTTT CTTTCTATGG	780
GTTTAAAAAC CAGATCCTGA GTGATATAGC ATTGGATGAC ATTAGCCTAA CATATGGGAT	840

TTGTAATATG AGTGTCTATC CAGAACCAAC TTTAGTCCCA ACTCCTCCAC CAGAACTTCC	900
CACGGACTGT GGAGGGCCTC ATGACCTGTG GGAGCCAAAT ACAACATTCA CGTCTATAAA	960
CTTCCCAAAC AGCTACCCTA ATCAGGCTTT CTGTATTTGG AATTTAAATG CACAAAAGGG	1020
AAAAAATATT CAGCTCCACT TTCAAGAATT TGACCTGGAA AATATTGCAG ATGTAGTTGA	1080
AATCAGAGAT GGTGAAGGAG ATGATTCCTT GTTCTTAGCT GTGTACACAG GCCCTGGTCC	1140
AGTAAACGAT GTGTTCTCAA CCACCAACCG AATGACTGTG CTTTTTATCA CTGATAATAT	1200
GCTGGCAAAA CAGGGATTTA AAGCAAATTT CACTACTGGC TATGGCTTGG GGATTCCAGA	1260
ACCCTGCAAG GAAGACAATT TTCAGTGCAA GGATGGGGAG TGTATTCCGC TGGTGAATCT	1320
CTGTGACGGT TTTCCACACT GTAAGGATGG CTCAGATGAA GCACACTGTG TGCGTCTCTT	1380
CAATGGCACG ACAGACAGCA GTGGTTTGGT GCAGTTCAGG ATCCAAAGCA TATGGCATGT	1440
AGCCTGTGCC GAGAACTGGA CAACCCAGAT CTCAGATGAT GTGTGTCAGC TGCTGGGACT	1500
AGGGACTGGA AACTCATCCG TGCCAACCTT TTCTACTGGA GGTGGACCAT ATGTAAATTT	1560
AAACACAGCA CCTAATGGCA GCTTAATACT AACGCCAAGC CAACAGTGCT TAGAGGATTCT	1620
ACTGATTTTG CTACAATGTA ACTACAAATC ATGTGGGAAA AAACTGGTGA CTCAAGAAGT	1680
TAGCCCGAAG ATTGTCGGAG GAAGTGA CTCAGAGAAGGA GCCTGGCCTT GGGTCGTTGC	1740
TCTGTATTTT GACGATCAAC AGGTCTGCGG AGCTTCTCTG GTGAGCAGGG ATTGGCTGGT	1800
GTCGGCCGCC CACTGCGTGT ACGGGAGAAA TATGGAGCCG TCTAAGTGA AAGCAGTGCT	1860
AGGCCTGCAT ATGGCATCAA ATCTGACTTC TCCTCAGATA GAACTAGGT TGATTGACCA	1920
AATTGTCATA AACCCACACT ACAATAAACG GAGAAAGAAC AATGACATTG CCATGATGCA	1980
TCTTGAAATG AAAGTGA ACTACAGATTA TATACAGCCT ATTTGTTTAC CAGAAGAAAA	2040
TCAAGTTTTT CCCCCAGGAA GAATTTGTTT TATTGCTGGC TGGGGGGCAC TTATATATCA	2100
AGGTTCTACT GCAGACGTAC TGCAAGAAGC TGACGTTCCC CTTCTATCAA ATGAGAAATG	2160
TCAACAACAG ATGCCAGAAT ATAACATTAC GGAAATATG GTGTGTGCAG GCTATGAAGC	2220
AGGAGGGGTA GATTCTTGTC AGGGGGATTTC AGGCGGACCA CTCATGTGCC AAGAAAACAA	2280
CAGATGGCTC CTGGCTGGCG TGACGTCATT TGGATATCAA TGTGCACTGC CTAATCGCCC	2340
AGGGGTGTAT GCCCGGGTCC CAAGGTTTAC AGAGTGGATA CAAAGTTTTC TACATTAGAG	2400
TGTTTCCAGA AACAAAGATG AAAATCAGGC AGTTTTCCCA TTTCACTTTA AGAAGCATGG	2460
AAATTGAGAG TTAATAAAT AATAATTTAT AAAAGTCTTG ATTCTTACCT AAGGCACTGA	2520
AATGCTACAA AAAAAAAAAA ACCGGAATTC AGCTTGGACT TAACCAGGCT GAACTTGCGG	2580
C	2581

(2) INFORMATION FOR SEQ ID NO: 2:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 798 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Ala Cys Asp Gly Arg Phe Leu Leu Thr Gly Ser Ser Gly Ser Phe
 1 5 10 15
 Glu Ala Leu His Tyr Pro Lys Pro Ser Asn Asn Thr Ser Ala Val Cys
 20 25 30
 Arg Trp Ile Ile Arg Val Asn Gln Gly Leu Ser Ile Gln Leu Asn Phe
 35 40 45
 Asp Tyr Phe Asn Thr Tyr Tyr Ala Asp Val Leu Asn Ile Tyr Glu Gly
 50 55 60
 Met Gly Ser Ser Lys Ile Leu Arg Ala Ser Leu Trp Ser Asn Asn Pro
 65 70 75 80
 Gly Ile Ile Arg Ile Phe Ser Asn Gln Val Thr Ala Thr Phe Leu Ile
 85 90 95
 Gln Ser Asp Glu Ser Asp Tyr Ile Gly Phe Lys Val Thr Tyr Thr Ala
 100 105 110
 Phe Asn Ser Lys Glu Leu Asn Asn Tyr Glu Lys Ile Asn Cys Asn Phe
 115 120 125
 Glu Asp Gly Phe Cys Phe Trp Ile Gln Asp Leu Asn Asp Asp Asn Glu
 130 135 140
 Trp Glu Arg Thr Gln Gly Ser Thr Phe Pro Pro Ser Thr Gly Pro Thr
 145 150 155 160
 Phe Asp His Thr Phe Gly Asn Glu Ser Gly Phe Tyr Ile Ser Thr Pro
 165 170 175
 Thr Gly Pro Gly Gly Arg Arg Glu Arg Val Gly Leu Leu Thr Leu Pro
 180 185 190
 Leu Asp Pro Thr Pro Glu Gln Ala Cys Leu Ser Phe Trp Tyr Tyr Met
 195 200 205
 Tyr Gly Glu Asn Val Tyr Lys Leu Ser Ile Asn Ile Ser Ser Asp Gln
 210 215 220
 Asn Met Glu Lys Thr Ile Phe Gln Lys Glu Gly Asn Tyr Gly Gln Asn
 225 230 235 240
 Trp Asn Tyr Gly Gln Val Thr Leu Asn Glu Thr Val Glu Phe Lys Val
 245 250 255
 Ser Phe Tyr Gly Phe Lys Asn Gln Ile Leu Ser Asp Ile Ala Leu Asp
 260 265 270
 Asp Ile Ser Leu Thr Tyr Gly Ile Cys Asn Met Ser Val Tyr Pro Glu
 275 280 285
 Pro Thr Leu Val Pro Thr Pro Pro Glu Leu Pro Thr Asp Cys Gly
 290 295 300
 Gly Pro His Asp Leu Trp Glu Pro Asn Thr Thr Phe Thr Ser Ile Asn
 305 310 315 320
 Phe Pro Asn Ser Tyr Pro Asn Gln Ala Phe Cys Ile Trp Asn Leu Asn
 325 330 335

Ala Gln Lys Gly Lys Asn Ile Gln Leu His Phe Gln Glu Phe Asp Leu
 340 345 350
 Glu Asn Ile Ala Asp Val Val Glu Ile Arg Asp Gly Glu Gly Asp Asp
 355 360 365
 Ser Leu Phe Leu Ala Val Tyr Thr Gly Pro Gly Pro Val Asn Asp Val
 370 375 380
 Phe Ser Thr Thr Asn Arg Met Thr Val Leu Phe Ile Thr Asp Asn Met
 385 390 395 400
 Leu Ala Lys Gln Gly Phe Lys Ala Asn Phe Thr Thr Gly Tyr Gly Leu
 405 410 415
 Gly Ile Pro Glu Pro Cys Lys Glu Asp Asn Phe Gln Cys Lys Asp Gly
 420 425 430
 Glu Cys Ile Pro Leu Val Asn Leu Cys Asp Gly Phe Pro His Cys Lys
 435 440 445
 Asp Gly Ser Asp Glu Ala His Cys Val Arg Leu Phe Asn Gly Thr Thr
 450 455 460
 Asp Ser Ser Gly Leu Val Gln Phe Arg Ile Gln Ser Ile Trp His Val
 465 470 475 480
 Ala Cys Ala Glu Asn Trp Thr Thr Gln Ile Ser Asp Asp Val Cys Gln
 485 490 495
 Leu Leu Gly Leu Gly Thr Gly Asn Ser Ser Val Pro Thr Phe Ser Thr
 500 505 510
 Gly Gly Gly Pro Tyr Val Asn Leu Asn Thr Ala Pro Asn Gly Ser Leu
 515 520 525
 Ile Leu Thr Pro Ser Gln Gln Cys Leu Glu Asp Ser Leu Ile Leu Leu
 530 535 540
 Gln Cys Asn Tyr Lys Ser Cys Gly Lys Lys Leu Val Thr Gln Glu Val
 545 550 555 560
 Ser Pro Lys Ile Val Gly Gly Ser Asp Ser Arg Glu Gly Ala Trp Pro
 565 570 575
 Trp Val Val Ala Leu Tyr Phe Asp Asp Gln Gln Val Cys Gly Ala Ser
 580 585 590
 Leu Val Ser Arg Asp Trp Leu Val Ser Ala Ala His Cys Val Tyr Gly
 595 600 605
 Arg Asn Met Glu Pro Ser Lys Trp Lys Ala Val Leu Gly Leu His Met
 610 615 620
 Ala Ser Asn Leu Thr Ser Pro Gln Ile Glu Thr Arg Leu Ile Asp Gln
 625 630 635 640
 Ile Val Ile Asn Pro His Tyr Asn Lys Arg Arg Lys Asn Asn Asp Ile
 645 650 655
 Ala Met Met His Leu Glu Met Lys Val Asn Tyr Thr Asp Tyr Ile Gln
 660 665 670
 Pro Ile Cys Leu Pro Glu Glu Asn Gln Val Phe Pro Pro Gly Arg Ile
 675 680 685

30

Cys Ser Ile Ala Gly Trp Gly Ala Leu Ile Tyr Gln Gly Ser Thr Ala
 690 695 700
 Asp Val Leu Gln Glu Ala Asp Val Pro Leu Leu Ser Asn Glu Lys Cys
 705 710 715 720
 Gln Gln Gln Met Pro Glu Tyr Asn Ile Thr Glu Asn Met Val Cys Ala
 725 730 735
 Gly Tyr Glu Ala Gly Gly Val Asp Ser Cys Gln Gly Asp Ser Gly Gly
 740 745 750
 Pro Leu Met Cys Gln Glu Asn Asn Arg Trp Leu Leu Ala Gly Val Thr
 755 760 765
 Ser Phe Gly Tyr Gln Cys Ala Leu Pro Asn Arg Pro Gly Val Tyr Ala
 770 775 780
 Arg Val Pro Arg Phe Thr Glu Trp Ile Gln Ser Phe Leu His
 785 790 795

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 27 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ile Val Gly Gly Ser Asp Ser Tyr Glu Gly Ala Trp Pro Trp Val Val
 1 5 10 15
 Ala Leu Tyr Phe Asp Asp Gln Gln Val Cys Gly
 20 25

(2) INFORMATION FOR SEQ ID NO: 4:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CTCGAATTCA TGTGGGGTCG A

21

(2) INFORMATION FOR SEQ ID NO: 5:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTCGAATTCA TGTGGGGAGG A

21

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Asp Gln Gln Val Cys Gly
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TCCAAGCTTC CCAACTGTGT C

21

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Tyr Glu Gly Ala Trp Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

32

(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
TAYGARGGNG CNTGGCC

(2) INFORMATION FOR SEQ ID NO: 10:

17

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 6 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Phe Asp Asp Gln Gln Val
1 5

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TCCAAGCTTA CTGTGTCTCA A

(2) INFORMATION FOR SEQ ID NO: 12:

21

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(x1) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ile Val Gly Gly Ser Asp Ser Arg Glu Gly Ala Trp Pro Trp Val Val
1 5 10 15
Ala Leu Tyr Phe Asp Asp Gln Gln Val Cys Gly Ala Ser Leu
20 25 30

(2) INFORMATION FOR SEQ ID NO: 13:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 24 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Glu Gly Ala Trp Pro Trp Val Val Ala Leu Tyr Phe Asp Asp Gln Gln
1 5 10 15
Val Cys Gly Ala Ser Leu Val Ser
 20

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Asp Trp Leu Val Ser Ala Ala His Cys Val Tyr Gly Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Phe Thr Glu Trp Ile Gln Ser Phe Leu His
1 5 10

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Ile Cys Ser Ile Ala Gly Trp Gly Ala Leu Ile Tyr Gln Gly Ser Thr
1 5 10 15
Ala Asp Val Leu Gln Glu Ala
 20

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 23 amino acids

- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Trp Leu Leu Ala Gly Val Thr Ser Phe Gly Tyr Gln Cys Ala Leu Pro
 1 5 10 15
 Asn Asn Pro Gly Val Tyr Ala
 20

(2) INFORMATION FOR SEQ ID NO: 18:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Asn Met Glu Pro Ser Lys
 1 5

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CGCGGATCCC CRTANACRCA RTGNGC

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CCGGAATTCT TGGGTCGTTG CTCTGTAT

(2) INFORMATION FOR SEQ ID NO: 21:

26

28

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CGCGGATCCA TACAGAGCAA CGACCCAA

28

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Leu Val Thr Gln Glu Val Ser Pro Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

Ala Phe Thr Thr Gly Tyr Gly Leu Gly Ile Pro Glu Pro
1 5 10

(2) INFORMATION FOR SEQ ID NO: 24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 13 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

Leu Phe Gly Thr Thr Asp Ser Ser Gly Leu Val Gln Phe
1 5 10

36

(2) INFORMATION FOR SEQ ID NO: 25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 23 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CTATAGACTG CTGGGTAGTC CCC

(2) INFORMATION FOR SEQ ID NO: 26:

23

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 41 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

ATAAGAATGC GGCCGCAAGT TCAGCCTGGT TAAGTCCAAG C

(2) INFORMATION FOR SEQ ID NO: 27:

41

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CCAAATACAG AAAGCCTGAT TAGGG

(2) INFORMATION FOR SEQ ID NO: 28:

25

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 33 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTAGGTCGAC CGTGAATGTT GTATTTGGCT CCC

33

(2) INFORMATION FOR SEQ ID NO: 29:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

Leu Ser Ile Asn Ile Ser Ser Asp Gln Asn Met Glu Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 30:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

Val Ser Phe Tyr Gly Phe Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 31:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

Gln Lys Glu Gly Asn Tyr Gly Gln Asn Trp Asn Tyr Gly Gln Val Thr
1 5 10 15
Leu Asn Glu Thr
20

(2) INFORMATION FOR SEQ ID NO: 32:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

38

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

Val Gly Leu Leu Thr Leu Pro
1 5

(2) INFORMATION FOR SEQ ID NO: 33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 5 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

Thr Ile Phe Gln Lys
1 5

WHAT IS CLAIMED IS:

1. A nucleic acid sequence comprising an isolated nucleic acid sequence encoding enterokinase activity.
- 5 2. The nucleic acid sequence of claim 1 encoding mammalian enterokinase.
3. The nucleic acid sequence of claim 1 encoding bovine enterokinase.
4. The nucleic acid sequence of claim 1 encoding human enterokinase.
- 10 5. The nucleic acid sequence of claim 1 selected from the group consisting of genomic DNA, complementary DNA, and synthetic DNA.
6. The nucleic acid sequence of claim 1 and comprising a sequence substantially as set forth in SEQ
15 ID NO:1.
7. A nucleic acid sequence comprising a nucleic acid sequence encoding enterokinase activity and comprising a sequence substantially duplicative of the sequence as set forth in SEQ ID NO:1 from nucleotide 1691 to nucleotide 2398.
- 20 8. A nucleic acid sequence comprising a nucleic acid sequence comprising SEQ ID NO:1.
9. A nucleic acid sequence comprising a nucleic acid sequence comprising nucleotides 1691 to 2398 of SEQ ID NO:1.
- 25 10. A nucleic acid sequence encoding enterokinase activity said nucleic acid sequence being selected from the group consisting of:
 - (a) a nucleic acid sequence substantially as set forth in SEQ ID NO:1,
 - (b) a nucleic acid sequence which hybridizes to (a) under stringent conditions, and
 - 30 (c) a nucleic acid sequence which, but for the redundancy of the genetic code, would hybridize to (a).
11. The nucleic acid sequence of claim 1, further comprising a second nucleic acid sequence encoding a member selected from the group consisting of a pre-region, pro-region, and a pre/pro region.

12. The nucleic acid sequence of claim 11, wherein said second nucleic acid sequence is a member selected from the group consisting of the pre/pro region of PACE, the pre/pro region of trypsinogen, and the pre/pro region of yeast α -factor.
- 5 13. The nucleic acid sequence of claim 1, further comprising a second nucleic acid sequence encoding a thioredoxin-like molecule.
14. A host cell transformed or transfected with a nucleic acid sequence of claim 1.
- 10 15. A nucleic acid sequence vector comprising a nucleic acid sequence according to claim 1.
16. The vector of claim 15 further comprising an expression control sequence operatively associated with said nucleic acid sequence.
- 15 17. The vector of claim 15 corresponding to plasmid pEK-2/GI734 designated as ATCC Deposit No. 69232.
18. An expression product of the nucleic acid sequence of claim 1.
- 20 19. A method for producing enterokinase activity comprising:
- (a) growing, in culture, the host cell of claim 13, and
 - (b) isolating from said host cell or said culture the polypeptide product of the expression of said nucleic acid sequence.
- 25 20. A method for the production of a protein having enterokinase activity comprising:
- (a) disposing a nucleic acid sequence according to claim 1 in a cell free transcription and translation system, and
 - (b) isolating from said system the polypeptide product of the expression of said nucleic acid sequence.
- 30 21. The polypeptide product of the *in vitro* or *in vivo* expression of a nucleic acid sequence of claim 1.
22. A synthetic peptide duplicative of a sequence of amino acids present in enterokinase and unique to enterokinase.
- 35

23. An antibody specifically immunoreactive with a polypeptide according to claim 21.
24. A method for treating disorders associated with low levels of enterokinase activity comprising the step of administering a polypeptide according to claim 21.
- 5 25. A method for treating disorders associated with low levels of enterokinase activity comprising the step of administering a nucleic acid sequence of claim 1.
- 10 26. A pharmaceutical composition for use in treating disorders associated with low levels of enterokinase activity comprising a therapeutically effective amount of enterokinase activity according to claim 21 in a pharmaceutically acceptable vehicle.
- 15 27. A method for cleaving proteins comprising the step of cleaving said protein with the expression product of claim 1.
28. A method for producing proteins from fusion proteins comprising the steps of:
- (a) growing, in culture, a host cell transformed or transfected with
 - (i) a nucleic acid sequence which encodes enterokinase activity and which upon expression is segregated into the periplasmic space, and
 - 20 (ii) one or more nucleic acid sequences which encode a fusion protein and an enterokinase cleavage site and which, upon expression are segregated to the cytoplasmic space,
 - (b) allowing said periplasmic space and said cytoplasmic space to co-mingle thereby,
 - (c) allowing said enterokinase activity to cleave said fusion protein, and
 - (d) resulting in protein production.

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FIGURE 1

1	CGGAGCTTGTGATGGAAGATTTTGTGACTGGATCTTCTGGGTCCTTTG	50
51	AGGCTCTGCATTATCCCAAGCCTTCTAATAATACAAGCGCTGTTTGTGCGG	100
101	TGGATTATACGTGTAAACCAAGGACTTTCATTCAACTGAACTTCGATTA	150
151	TTTAAATACATATTATGCAGATGTATTAAATATTTATGAAGGAATGGGTT	200
201	CAAGCAAGATTTTAAGAGCTTCTCTCTGGTCAAATAATCCTGGCATAATT	250
251	AGGATTTTTTCCAATCAAGTTACTGCCACTTTTCTTATACAGTCTGATGA	300
301	AAGTGATTATATTGGCTTCAAAGTAACATACACTGCATTTAACAGCAAAG	350
351	AGCTTAATAATTATGAGAAAATCAACTGTAATTTTGAAGATGGCTTCTGT	400
401	TTCTGGATCCAGGATCTAAATGATGACAATGAGTGGGAAAGGACTCAGGG	450
451	AAGCACCTTTCCTCCATCTACTGGACCAACTTTTGACCACACTTTTGGCA	500
501	ATGAGTCAGGATTTTACATTTCCACCCCAACTGGACCAGGAGGAAGACGA	550
551	GAAAGAGTAGGACTTTTAACTCTCCCTTTAGATCCCACTCCTGAACAAGC	600
601	CTGCCTTAGTTTCTGGTATTATATGTATGGTGAAAATGTTTACAAACTAA	650
651	GCATTAATATCAGCAGTGACCAAAACATGGAGAAGACAATTTTCCAAAAA	700
701	GAAGGAAATTATGGACAAAATTGGAACATATGGACAAGTAACATTAAATGA	750
751	AACAGTGGAATTTAAGGTTTCTTTCTATGGGTTTAAAAACCAGATCCTGA	800
801	GTGATATAGCATTGGATGACATTAGCCTAACATATGGGATTTGTAATATG	850
851	AGTGTCTATCCAGAACCAACTTTAGTCCCAACTCCTCCACCAGAACTTCC	900
901	CACGGACTGTGGAGGGCCTCATGACCTGTGGGAGCCAAATACAACATTCA	950
951	CGTCTATAAACTTCCCAAACAGCTACCCTAATCAGGCTTTCTGTATTTGG	1000
1001	AATTTAAATGCACAAAAGGGAAAAAATATTCAGCTCCACTTTCAAGAATT	1050
1051	TGACCTGGAAAATATTGCAGATGTAGTTGAAATCAGAGATGGTGAAGGAG	1100

SUBSTITUTE SHEET (RULE 26)

FIGURE 1A

1101 ATGATTCCTTGTTCCTTAGCTGTGTACACAGGCCCTGGTCCAGTAAACGAT 1150
1151 GTGTTCTCAACCACCAACCGAATGACTGTGCTTTTATCACTGATAATAT 1200
1201 GCTGGCAAACAGGGATTTAAAGCAAATTTCACTACTGGCTATGGCTTGG 1250
1251 GGATTCCAGAACCCTGCAAGGAAGACAATTTTCAGTGCAAGGATGGGGAG 1300
1301 TGTATTCCGCTGGTGAATCTCTGTGACGGTTTTCCACACTGTAAGGATGG 1350
1351 CTCAGATGAAGCACACTGTGTGCGTCTCTTCAATGGCACGACAGACAGCA 1400
1401 GTGGTTTGGTGCAGTTCAGGATCCAAAGCATATGGCATGTAGCCTGTGCC 1450
1451 GAGAACTGGACAACCCAGATCTCAGATGATGTGTGTCAGCTGCTGGGACT 1500
1501 AGGGACTGGAACTCATCCGTGCCAACCTTTTCTACTGGAGGTGGACCAT 1550
1551 ATGTAAATTTAAACACAGCACCTAATGGCAGCTTAATACTAACGCCAAGC 1600
1601 CAACAGTGCTTAGAGGATTCAGTATTTTGCTACAATGTAAC TACAATC 1650
1651 ATGTGGGAAAAAAGTGGTGAAGTCAAGAGTTAGCCCGAAGATTGTCGGAG 1700
1701 GAAGTGACTCCAGAGAAGGAGCCTGGCCTTGGGTCGTTGCTCTGTATTTT 1750
1751 GACGATCAACAGGTCTGCGGAGCTTCTCTGGTGAGCAGGGATTGGCTGGT 1800
1801 GTCGGCCGCCCACTGCGTGTACGGGAGAAATATGGAGCCGTCTAAGTGGA 1850
1851 AAGCAGTGCTAGGCCTGCATATGGCATCAAATCTGACTTCTCCTCAGATA 1900
1901 GAACTAGGTTGATTGACCAAATTGTCATAAACCCACACTACAATAAACG 1950
1951 GAGAAAGAACAATGACATTGCCATGATGCATCTTGAAATGAAAGTGAAGT 2000
2001 ACACAGATTATATACAGCCTATTTGTTTACCAGAAGAAAATCAAGTTTTT 2050
2051 CCCCAGGAAGAATTTGTTCTATTGCTGGCTGGGGGGCACTTATATATCA 2100
2101 AGGTTCTACTGCAGACGTACTGCAAGAAGCTGACGTCCCTTCTATCAA 2150
2151 ATGAGAAATGTCAACAACAGATGCCAGAATATAACATTACGGAAAATATG 2200
2201 GTGTGTGCAGGCTATGAAGCAGGAGGGGTAGATTCTTGTGAGGGGGATTC 2250

SUBSTITUTE SHEET (RULE 26)

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FIGURE 1B

2251 AGGCGGACC·ACTCATGTGCCAAGAAAACA·ACAGATGGCTCCTGGCTGGCG· 2300
2301 TGACGTCATT·TGGATATCAATGTGCACTGCCTAATCGCCCAGGGGTGTAT· 2350
2351 GCCCGGGTCCCAAGGTTCA·CAGAGTGGATACAAAGTTTCTACATTAGAG· 2400
2401 TGTTTCCAGAA·ACAAAGATGAAAATCAGGCAGTTTTCCCATTTCACTTTA· 2450
2451 AGAAGCATGGAAAT·TGAGAGTTAAAAAATAATAATTTATAAAAGTCTTG· 2500
2501 ATTCTTACCTAAGGCACTGAAATGCTACAAAAAAAAAAAA·ACCGGAATTC 2550
2551 AGCTTGGA·CTTAACCAGGCTGAACTTGCGGC 2581

SUBSTITUTE SHEET (RULE 26)

FIGURE 2

10 30 50
GACDGRFLLTGSSGSFEALHYPKPSNNTSAVCRWIIRVNQGLSIQLNPDYFNTYYADVLN
70 90 110
IYEGMGSSKILRASLWSNNPGIIRIFSNQVTATFLIQSDSDYIGFKVITYTAFNSKELNN
130 150 170
YEKINC�FEDGFCFWIQDLNDDNEWERTQGSTFPPSTGPTFDHTFGNESGFYISTPTGPG
190 210 230
GRRERVGLLTLPLDPTPEQACLSEFWYMYGENVYKLSINISSDQNMECTIFQKEGNYGQN
250 270 290
WNYGQVTLNETVEFKVSFYGFKNQILSDIALDDISLTYGICNMSVYPEPTLVPTPPPELP
310 330 350
TDCGGPHDLWEPNTTFTSINFPNSYPNQAFCIWNLNQKGNQIQLHFQEPDLENIADVVE
370 390 410
IRDGEGDDSLFLAVYTGPVPNDVFPSTTNRMTVLFITDNMLAKQGFKANFTTGYGLGIPE
430 450 470
PCKEDNFQCKDGECIPLVNLCDGFPHCKDGSDEAHCVRLFNGTTDSSGLVQFRIQSIWHV
490 510 530
ACAENWTTQISDDVCQLLGLGTGNSSVPTFSTGGGPYVNLNTAPNGSLILTPSQQCLEDSE
550 570 590
LILLQCNYSKCGKKLVTQEVSPKIVGGSDSREGAWPWVVALYFDDQQVCGASLVSRDWLV
610 630 650
SAAHCVYGRNMEPSKWKAVLGLHMASNLTSPIETRLIDQIVINPHYNKRRKNNDIAMMH
670 690 710
LEMKVNYTDYIQPICLPEENQVFPFGRICSIAGWGALIYQGSTADVLQEADVPLLSNEKC
730 750 770
QQQMPEYNITENMVCAGYEAGGVDSQGDSEGGPLMCQENNRWLLAGVTSFGYQCALPNRP
790
GVYARVPRFTEWISFLH*

INTERNATIONAL SEARCH REPORT

Initial Application No

PCT/US 94/00616

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C12N15/57 C12N9/64 C12N15/62 C07K15/28 A61K37/547

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHEMICAL ABSTRACTS, vol. 116, no. 5, 3 February 1992, Columbus, Ohio, US; abstract no. 36904a, LIGHT ALBERT ET AL. 'The amino-terminal sequence of the catalytic subunit of bovine enterokinase.' page 333 ; see abstract & J. PROTEIN CHEM. vol. 10, no. 5 , 1991 pages 475 - 480 --- -/--	1

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

* Special categories of cited documents :

- 'A' document defining the general state of the art which is not considered to be of particular relevance
- 'E' earlier document but published on or after the international filing date
- 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- 'O' document referring to an oral disclosure, use, exhibition or other means
- 'P' document published prior to the international filing date but later than the priority date claimed

- 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- '&' document member of the same patent family

Date of the actual completion of the international search

10 May 1994

Date of mailing of the international search report

26-05-1994

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax (+31-70) 340-3016

Authorized officer

Delanghe, L

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/00616

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		Relevant to claim No.
Category	Citation of document, with indication, where appropriate, of the relevant passages	
Y	CHEMICAL ABSTRACTS, vol. 102, no. 3, 21 January 1985, Columbus, Ohio, US; abstract no. 20188v, J.R. THOMAS ET AL. 'Purification of bovine and porcine enterokinase by affinity chromatography with immobilized kidney bean enterokinase inhibitor.' page 303 ; see abstract & J.BIOSCI. vol. 6, no. 3 , 1984 , INDIA pages 289 - 295	1
Y	--- JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 259, no. 21 , 1984 , BALTIMORE US pages 13195 - 13198 ALBERT LIGHT ET AL. 'The preparation and properties of the catalytic subunit of bovine enterokinase.' see the whole document	1
Y	--- WO,A,84 04301 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 8 November 1984 see claims	1
X	--- CHEMICAL ABSTRACTS, vol. 113, no. 25, 17 December 1990, Columbus, Ohio, US; abstract no. 229053u, MIYOSHI YASUYUKI ET AL 'Monoclonal antibody against human enterokinase and immunohistochemical localization of the enzyme.' page 515 ; see abstract & GASTROENTEROL. JPN. vol. 25, no. 3 , 1990 page 320-327	23
X	--- DATABASE WPI Section Ch, Week 6800, Derwent Publications Ltd., London, GB; Class B, AN 66-29739F & CA,A,769 697 (ARMOUR PHARM) see abstract	24-26
P,X	--- JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 268, no. 31 , 5 November 1993 , BALTIMORE US pages 23311 - 23317 E.R. LA VALLIE ET AL. 'Cloning and functional expression of a cDNA encoding the catalytic subunit of bovine enterokinase.' see the whole document	1-28
1	-----	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/00616

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark : Although claims 24-25 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/00616

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8404301	08-11-84	AU-A- 2868684	19-11-84
		EP-A- 0149615	31-07-85
		US-A- 4593018	03-06-86

Form PCT/ISA/210 (patent family annex) (July 1992)

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